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(54) Title: METHODS OF MAKING AN RNP PARTICLE HAVING NUCLEOTIDE INTEGRASE ACTIVITY

(57) Abstract

Methods for preparing nucleotide integrases are provided. The nucleotide integrases are prepared by combining in vitro an excised, group II intron RNA, referred to hereinafter as "exogenous RNA", with a group II intron–encoded protein. The exogenous RNA is prepared by in vitro transcription of a DNA molecule which comprises a group II intron sequence.

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METHODS OF MAKING AN RNP PARTICLE HAVING NUCLEOTIDE INTEGRASE ACTIVITY

BACKGROUND

Nucleotide integrases are molecular complexes that are capable of cleaving nucleic acid substrates at specific recognition sites and of concomitantly inserting nucleic acid molecules into the nucleic acid substrate at the cleavage site. Thus, nucleotide integrases are useful tools, particularly for genome mapping, genetic engineering and disrupting the synthesis of gene products. Structurally, nucleotide integrases are ribonucleoprotein (RNP) particles that comprise an excised, group II intron RNA and a group II intron-encoded protein, which is bound to the group II intron RNA.

Conventionally, nucleotide integrases are made by isolating RNP particles that have nucleotide integrase activity from source organisms which comprise a DNA molecule that encodes both the RNA and protein subunits of the nucleotide integrase. In order to obtain nucleotide integrases other than wild type, the source organisms are mutagenized. The mutagenesis is a laborious, multistep process. Moreover, this process yields limited quantities of the nucleotide integrase.

Accordingly, it is desirable to have methods for making nucleotide integrases which are not laborious and which permit the nucleotide integrase to be readily modified from the wild type. Methods which yield at least microgram quantities of substantially pure nucleotide integrases are especially desirable.

SUMMARY OF THE INVENTION

The present invention provides new, improved, and easily manipulable methods for making nucleotide integrases.

In one embodiment, the nucleotide integrase is prepared by introducing a DNA molecule which comprises a group II intron DNA sequence into a host cell. Preferably the DNA molecule further comprises a sequence which encodes a tag that facilitates isolation of RNP particles having nucleotide integrase activity from the host cell. Preferably, the tag sequence is linked to the open reading frame (ORF) sequence of the group II intron DNA. The group II intron DNA sequence is then expressed in the host cell such that RNP particles having nucleotide integrase activity are formed in the cell. Such RNP particles comprise an excised group II intron RNA molecule and a group II intron- ncoded protein, both of which

are encoded by the introduced DNA molecule. Thereafter, the RNP particles having nucleotide integrase activity are isolated from the cell.

In another embodiment, the nucleotide integrase is prepared by combining in vitro an excised, group II intron RNA, referred to hereinafter as "exogenous RNA", with a group II intron-encoded protein. The exogenous RNA is prepared by in vitro transcription of a DNA molecule which comprises a group II intron sequence. The group II intron-encoded protein is made by introducing into a host cell a DNA molecule that comprises at least the open reading frame sequence of a group II intron and then expressing the open reading frame sequence in the host cell. The DNA molecule may comprise the open reading frame sequence operably linked to a promoter, preferably an inducible promoter. Thereafter, the cell is fractionated and the protein is recovered and combined in vitro with the exogenous RNA to provide RNP particles having nucleotide integrase activity. Alternatively, the DNA molecule may comprise a group II intron sequence that encodes both a group II intron RNA as well as a group II intron encoded protein. The DNA molecule is then expressed in the host cell to provide RNP particles that comprise the group II intron-encoded protein bound to the group II intron RNA. Thereafter, the RNP particles comprising the group II intron-encoded protein and the group II intron RNA are isolated from the cell and treated with a nuclease to remove the RNA and to provide the group II-intron encoded protein. The group II intron-encoded protein is then combined in vitro with the exogenous RNA to provide RNP particles having nucleotide integrase activity.

The present invention also relates to isolated RNP particles having nucleotide integrase activity.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 depicts the interaction at the target site between the EBS1 and EBS2 sequences of the group II intron RNA 2 of the *S. cerevisiae* mitochondrial *COX1* gene, hereinafter referred to as the "aI2" RNA, with the IBS1 and IBS2 sequences of a nucleic acid substrate. The cleavage site is represented by an arrow.

Figure 2 is a schematic representation of the domains in three representative group II-intron encoded proteins, namely the protein which is encoded by the ORF sequence of the group II intron 2 of the *S. cerevisiae* mitochondrial *COX1* gene, the group II intron 2 of *the*

M. polymorpha mitochondrial *COX1* gene, and the group II intron 1 of the *N. tabacum* chloroplast *trnK* gene.

Figure 3 is the plasmid map of pETLtrAl9.

Figure 4 shows the nucleotide sequence of the 2.8 kb HindIII fragment that is present in pETLtrAl9 and that includes the Ll.ltrB intron DNA sequence and portions of the nucleotide sequence of the flanking exons *ltrBEl* and *ltrBE2,* SEQ. ID. *NO.* 1., the nucleotide sequence of the LtrA open reading frame, SEQ. ID. *NO.* 2, and the amino acid sequence of the LtrA protein, SEQ. ID. *NO.* 3.

Figure 5 is the plasmid map of plasmid pETLtrAl-1.

Figure 6 is a schematic representation of the inserts in pLE12, pETLtrAl9 and pETLtrAl-1.

Figure 7A is the sequence of the sense strand of a double-stranded DNA substrate, SEQ. ID. NO. 4, which is cleaved by RNP particles that comprise a wild-type excised, Ll.ltrB intron RNA and an LtrA protein. Figure 7B is the sequence of the sense strand of a double stranded DNA substrate which is cleaved by RNP particles that comprise an excised Ll.ltrB intron RNA having a modified EBS1 sequence and an LtrA protein.

Figure 8a is a schematic depiction of the substrate which is cleaved by RNP particles comprising the wild-type Ll.ltrB intron RNA and the LtrA protein, and Figure 8b shows the IBS1 and IBS2 sequences of the substrate and the cleavage sites of the double-stranded DNA substrate which is cleaved by these RNP particles.

DETAILED DESCRIPTION OF THE INVENTION

Nucleotide Integrases

Functionally, nucleotide integrases are endonucleases that are capable of cleaving nucleic acid substrates at specific recognition sites and of concomitantly inserting nucleic acid molecules into the substrate at the cleavage site. Structurally, nucleotide integrases are ribonucleoprotein (RNP) particles that comprise an excised, group II intron RNA and a group II intron-encoded protein, which is bound to the excised group II intron RNA. "Excised group II intron RNA," as used herein, refers to the RNA that is, or that is derived from, an *in vitro* or *in vivo* transcript of the group II intron DNA and that lacks flanking exon sequences at the 5' end and the 3' end of the intron sequence. The excised, group II intron RNA typically has six domains and a characteristic secondary and tertiary structure, which is

shown in Saldahana et al., 1993, Federation of the American Society of Experimental Biology Journal, Vol 7 p15-24, which is specifically incorporated herein by reference. Domain IV of the group II intron RNA contains the open reading frame ("ORF") nucleotide sequence which encodes the group II intron encoded protein. The excised group II intron RNA also has two sequences in domain I which are capable of hybridizing with two sequences in the target site of the intended nucleic acid substrate. The first sequence, referred to hereinafter as the "EBS1" sequence, is capable of hybridizing with a sequence, referred to hereinafter as the "IBS1" sequence, which is immediately upstream of the cleavage site in the substrate. The second sequence, referred to hereinafter as the "EBS2" sequence, is capable of hybridizing with a sequence, hereinafter referred to as the "IBS2" sequence, which is upstream of the IBS1 sequence.

The excised group II intron RNA has a wild-type sequence, i.e. a sequence which is identical to the sequence of a group II intron RNA that is found in nature, or the excised group II intron RNA has a modified sequence, i.e. a sequence which is different from the sequence of group II intron RNA molecules that are found in nature. For nucleotide integrases in which the group II intron RNA has a wild-type sequence, the EBS1 sequence typically is complementary to a sequence of about 5-7 nucleotides, hereinafter referred to as the "first set", which is located at the 3' end of the exon that is joined to the 5' end of the intron in the gene. Similarly, the EBS2 sequence of the wild-type group II intron RNA typically is complementary to a sequence of about 5-7 nucleotides in the 5' exon, hereinafter referred to as the "second set", which is upstream, typically immediately upstream, of the first set. Thus, the EBS1 and EBS2 sequences of a wild-type group II intron RNA can usually be predicted by finding sequences in domain I of the intron that are complementary to the first set and second set of nucleotides in the 5' exon.

In the wild-type group II intron RNA of the *Lactococcus lactis ltrB* gene, hereinafter referred to as the wild-type Ll.ltrB intron RNA, EBS1 comprises 7 nucleotides, is located at position 3132-3138 (numbered according to Mills et al., 1996, J. Bact., 178, 3531-3538), and has the sequence GUUGUGG. EBS2 of the wild-type Ll.ltrB intron RNA comprises 6 nucleotides, is located at positions 3076-3081 and has the sequence AUGUGU. In the wild-type group II intron RNA 1 of the *S. cerevisiae* mitochondrial *COX1* gene, hereinafter referred to as the "wild-type aI1 RNA", EBS1 comprises 6 nucleotides, is located at position 426-431 (numbered according to Bonitz et al., 1980, J. Biol. Chem.: 255, 11927-11941), and has the

sequence CGUUGA. EBS2 of the wild-type aI1 RNA comprises 6 nucleotides, is located at positions 376-381 and has the sequence ACAAUU. In the wild-type group II intron RNA 2 of the *S. cerevisiae* mitochondrial *COX1* gene, hereinafter referred to as the "wild-type aI2" RNA, EBS1 comprises 6 nucleotides, is located at position 2985-2990 (numbered according to Bonitz et al., 1980, J. Biol. Chem.: 255, 11927-11941) and has the sequence AGAAGA. EBS2 of the wild-type aI2 RNA comprises 7 nucleotides, is located at positions 2935-29410, and has the sequence UCAUUAA. The interaction between EBS1 and EBS2 of the wild-type aI2 RNA with its intended substrate is depicted in Figure 1.

The excised group II intron RNA may also have a sequence different from a group II intron RNA that is found in nature, and thus be a modified, excised group II intron RNA. Modified excised group II intron RNA molecules, include, for example, group II intron RNA molecules that have nucleotide base changes or additional nucleotides in the internal loop regions of the group II intron RNA, preferably the internal loop region of domain IV and group II intron RNA molecules that have nucleotide base changes in the sequences of EBS1 and/or EBS2. Nucleotide integrases in which the group II intron RNA has nucleotide base changes in the sequences of EBS1 or EBS2, as compared to the wild type, typically have altered specificity for the intended nucleic acid substrate.

The group II intron-encoded protein has an X domain, a reverse transcriptase domain, and, preferably, a Zn domain. The X domain of the protein has a maturase activity. The Zn domain of the protein has Zn^{2+} finger-like motifs. As used herein, a group II intron-encoded protein includes modified group II intron-encoded proteins that have additional amino acids at the N terminus, or C terminus, or alterations in the internal regions of the protein as well as wild-type group II intron-encoded proteins. The domains of three representative group II intron-encoded proteins are depicted in Figure 2.

The RNP particles having nucleotide integrase activity cleave single-stranded RNA molecules, single-stranded DNA molecules, and double-stranded DNA molecules. The RNP particles having nucleotide integrase activity also insert the group II intron RNA subunit of the RNP particle into the cleavage site. Thus, RNP particles having nucleotide integrase activity both cleave nucleic acid substrates and insert nucleic acid molecules into the cleavage site. With double-stranded DNA substrates, the nucleotide integrase inserts the group II intron RNA into the first strand, i.e., the strand that contains the IBS1 and IBS2 sequences, of the cleaved DNA substrate and, preferably, a cDNA molecule into the second strand of the

cleaved DNA substrate. The excised group II intron RNA subunit of the nucleotide integrase catalyzes cleavage of the single-stranded-substrates and the first strand of the double-stranded DNA substrate. The cleavage that is catalyzed by the excised group II intron RNA also results in the insertion, either partially or completely, of the excised group II intron RNA into the cleavage site, i.e. between nucleotide +1, which is immediately downstream of the cleavage site, and nucleotide -1, which is immediately upstream of the cleavage site. The group II intron-encoded protein subunit catalyzes cleavage of the second strand of the double-stranded DNA substrate. The second strand of the double stranded DNA substrate is cut at a position from about 9 to about 11 base pairs downstream of the cleavage site in the first strand, i.e. at a site between nucleotide positions +9, +10, and +11. It is believed that the group II intron-encoded protein also assists cleavage of the first strand of the double stranded DNA substrate by stabilizing the group II intron RNA. Thus, the RNP particle having nucleotide integrase activity is active under conditions that are similar to physiological conditions.

To cleave the substrates, it is preferred that the EBS1 and EBS2 sequences of the group II intron RNA of the nucleotide integrase have at least 90% complementarity, preferably full complementarity, with the IBS1 and IBS2 sequences, respectively, of the intended substrate. Thus, if there is not at least 90% complementarity between the EBS sequences of the excised group II intron RNA and IBS sequences of the intended substrate, it is preferred that nucleotide base changes be made in the non-complementary EBS sequences. To cleave single-stranded and double-stranded nucleic acid substrates efficiently, it is preferred that the nucleotide delta, which immediately precedes the first nucleotide of EBS1 be complementary to the nucleotide at +1 in the target site. Thus, if the delta nucleotide is not complementary to the nucleotide at +1 in the target site, the group II intron RNA is modified to contain a delta nucleotide which is complementary to the nucleotide at +1 on the sense strand of the substrate. To cleave double stranded DNA substrates efficiently, it is preferred that the target site has a sequence that is recognized by the group II intron-encoded protein of the nucleotide integrase. For example, cleavage of a double-stranded DNA substrate is achieved with a nucleotide integrase comprising a wild-type Ll.ltrB RNA and LtrA protein if the first strand of the substrate contains the sequence,

5'-TCGATCGTGAACACATCCATAACC'3', SEQ.ID.NO.___. which represents the sequence from -23 to +1 in the target site of the first strand.

A. Preparation of the Nucleotide Integrase by Isolation from a Genetically-Engineered Cell.

In one embodiment, RNP particles having nucleotide integrase activity are made by introducing an isolated DNA molecule which comprises a group II intron DNA sequence into a host cell. Preferably, the DNA molecule further comprises an IBS1 sequence and an IBS2 sequence just upstream of the 5' end of the group II intron DNA sequence to allow splicing of the group II intron RNA from a transcript of the group II intron DNA sequence. Suitable DNA molecules include, for example, viral vectors, plasmids, and linear DNA molecules. Following introduction of the DNA molecule into the host cell, the group II intron DNA sequence is expressed in the host cell such that excised RNA molecules encoded by the introduced group II intron DNA sequence and protein molecules encoded by introduced group II intron DNA sequence are formed in the cell. The excised group II intron RNA and group II intron-encoded protein are combined within the host cell to produce an RNP particle having nucleotide integrase activity.

Preferably, the introduced DNA molecule also comprises a promoter, more preferably an inducible promoter, operably linked to the group II intron DNA sequence. Preferably, the DNA molecule further comprises a sequence which encodes a tag to facilitate isolation of the RNP particles having nucleotide integrase activity, such as, for example, an affinity tag and/or an epitope tag. Preferably, the tag sequences are at the 5' or 3' end of the open reading frame sequence. Suitable tag sequences include, for example, sequences which encode a series of histidine residues, the Herpes simplex glycoprotein D, i.e., the HSV antigen, or glutathione S-transferase. An especially suitable tag is a sequence which encodes the intein from the *S. cerevisiae* VMA1 gene linked to the chitin binding domain from *Bacillus circulans*. Typically, the introduced DNA molecule also comprises nucleotide sequences that encode a replication origin and a selectable marker. Optionally, the introduced DNA molecule comprises sequences that encode molecules that modulate expression, such as for example T7 lysozyme.

The DNA molecule comprising the group II intron sequence is introduced into the host cell by conventional methods, such as, by cloning the DNA molecule into a vector and by introducing the vector into the host cell by conventional methods, such as electroporation or by $CaCl_2$-mediated transformation procedures. The method used to introduce the DNA molecule depends on the particular host cell used. Suitable host cells are those which are

capable of expressing the group II intron DNA sequence. Suitable host cells include, for example, heterologous or homologous bacterial cells, yeast cells, mammalian cells, and plant cells. In those instances where the host cell genome and the group II intron DNA sequence use different genetic codes, it is preferred that the group II intron DNA sequence be modified to comprise codons that correspond to the genetic code of the host cell. The group II intron DNA sequence, typically, is modified by using a DNA synthesizer or by in vitro site directed mutagenesis, such as by PCR mutagenesis, to prepare a group II intron DNA sequence with different codons. Alternatively, to resolve the differences in the genetic code of the intron and the host cell, DNA sequences that encode the tRNA molecules which correspond to the genetic code of the group II intron are introduced into the host cell. Optionally, DNA molecules which comprise sequences that encode factors that assist in RNA or protein folding, or that inhibit RNA or protein degradation are also introduced into the cell.

The DNA sequences of the introduced DNA molecules are then expressed in the host cell to provide a transformed host cell. As used herein the term "transformed cell" means a host cell that has been genetically engineered to contain and express additional DNA, primarily heterologous DNA, and is not limited to cells which are cancerous. Then the RNP particles having nucleotide integrase activity are isolated from the transformed host cells.

The RNP particles having nucleotide integrase activity are isolated, preferably by lysing the transformed cells, such as by mechanically and/or enzymatically disrupting the cell membranes of the transformed cell. Then the cell lysate is fractionated into an insoluble fraction and soluble fraction. Preferably, an RNP particle preparation is isolated from the soluble fraction. The RNP particle preparations include the RNP particles having nucleotide integrase activity as well as ribosomes, mRNA and tRNA molecules. Suitable methods for isolating RNP particle preparations include, for example, centrifugation of the soluble fraction through a sucrose cushion. The RNP particles, preferably, are further purified from the RNP particle preparation or from the soluble fraction by, for example, separation on a sucrose gradient, or a gel filtration column, or by other types of chromatography. For example, in those instances where the group II-intron encoded protein subunit of the desired RNP particle has been engineered to include a tag, the RNP particles having nucleotide integrase activity are purified from the particle preparation by affinity chromatography on a matrix which recognizes and binds to the tag. For example, NiNTA SuperflowTH from Qiagen, Chatsworth CA, is suitable for isolating RNP particles having nucleotide integrase

activity when the group II intron-encoded protein has a histidine tag. It has been found that the a system which employs a chitin column and an intein and chitin binding domain tag on the group II intron-encoded protein results in the production of RNP particles that are substantially pure, i.e., the intron encoded protein represents at least 95% of the protein in the RNP particles eluted from the column. Thus, the latter system is particularly suitable for isolating RNP particles having nucleotide integrase activity.

B. Preparation of the Nucleotide Integrase by Combining Exogenous RNA with a Group II Intron-Encoded Protein to Form a Reconstituted RNP Particle

In another embodiment, the nucleotide integrase is formed by combining an isolated exogenous RNA with an isolated group II intron-encoded protein in vitro to provide a reconstituted RNP particle having nucleotide integrase activity. The exogenous RNA is made by in vitro transcription of the group II intron DNA. The exogenous RNA may be made by in vitro transcription of the group II intron DNA only, i.e. the transcript lacks flanking exon sequences. Alternatively, the exogenous RNA is made by in vitro transcription of the group II intron DNA and the DNA of all, or portions, of the flanking exons to produce an unprocessed transcript which contains the group II intron RNA and the RNA encoded by the flanking exons or portions thereof. Then the exogenous RNA is spliced from the unprocessed transcript.

The purified group II intron-encoded protein is prepared by introducing into a host cell an isolated DNA molecule that comprises at least the open reading frame sequence of a group II intron. The DNA molecule may comprise a group II intron ORF sequence operably linked to an inducible promoter. Alternatively, the DNA molecule may comprise a group II intron DNA sequence. Preferably, the introduced DNA molecule also comprises a sequence at the 5' or 3' end of the group II intron ORF sequence which, when expressed in the host cell, provides an affinity tag or epitope on the N-terminus or C-terminus of the group II intron-encoded protein. Thus, the DNA molecule may comprise at the 5' or 3' end of the ORF, for example, a sequence which encodes a series of histidine residues, or the HSV antigen, glutathione-S-transferase, or an intein linked to a chitin binding domain. Typically, the DNA molecule also comprises nucleotide sequences that encode a replication origin and a selectable marker.

When the introduced DNA molecules comprise a group II intron ORF sequence operably linked to an inducible promoter, the ORF sequence is then expressed in the host cell, preferably by adding a molecule which induces expression, to provide a host cell that contains RNP particles comprising the group II intron-encoded protein associated with endogenous nucleic acids, particularly endogenous RNA molecules. Then the transformed cell is lysed, and preferably fractionated into a soluble fraction and an insoluble fraction. The RNP particles comprising the protein and the endogenous RNA are then isolated, preferably from the soluble fraction, preferably by using methods such as affinity chromatography. The RNP particles are then incubated with the exogenous RNA, preferably in a buffer, to allow the exogenous RNA to displace the associated RNA molecules and to form RNP particles having nucleotide integrase activity. Optionally, the RNP particles, are treated with a nuclease to remove the RNA that is associated with the group II intron encoded protein prior to incubation of the protein preparation with the exogenous RNA. The RNP particles may be treated with the nuclease by adding the nuclease to the soluble fraction. Alternatively, the RNP particles may be treated with the nuclease after isolation of the RNP particles from the soluble fraction.

When DNA molecules comprise a splicing-competent group II intron sequence, are introduced and expressed in the host cells, RNP particles comprising a group II intron-encoded protein associated with an excised group II intron RNA that encodes the protein are produced. When DNA molecules comprise a splicing-defective group II intron sequence, are introduced and expressed in the host cells, the group II intron-encoded protein is not associated with an excised, group II intron RNA that encodes the protein The RNP particles that are produced when a splicing-defective group II intron DNA sequence is introduced and expressed in a host cell comprise other types of RNA molecules, such as for example, unspliced group II intron RNA molecules that encode the protein, ribosomal RNA molecules, mRNA molecules, tRNA molecules or other nucleic acids. Following formation of the RNP particles in the host cell, the transformed cell is lysed, and preferably fractionated into a soluble fraction and an insoluble fraction. The RNP particles comprising the protein are then isolated, preferably from the soluble fraction, preferably by using methods such as affinity chromatography. The isolated RNP particles are then treated with a nuclease that degrades all of the endogenous RNA molecules. Preferably the RNP particles are treated with a nuclease which can be chemically inactivated, such as for example, micrococcal nuclease. The group

II intron-encoded protein preparation is then combined with the exogenous RNA, preferably in a buffer, to allow formation of RNP particles having nucleotide integrase activity

These methods enable production of increased quantities of nucleotide integrases. Conventional methods produce approximately 0.1 to 1 μg of an RNP particles having nucleotide integrase per liter of cultured cells. However, these RNP particles are highly contaminated with other proteins. The methods of the present invention enable the production of at least 0.5 mg of RNP particles having nucleotide integrase activity per liter of cultured cells. Moreover, the RNP particles having nucleotide integrase activity produced in accordance with the present methods are substantially pure, i.e., at least 95% of the protein in the final RNP particle preparation is the group II intron-encoded protein. The present methods also offer the further advantage of permitting the sequences of the RNA component and the protein component of the nucleotide integrase to be readily modified. Typically, the nucleotide integrases are modified by introducing nucleotide base changes, deletions, or additions into the group II intron RNA by PCR mutagenesis of the group II intron.

The following examples of methods for preparing a group II intron-encoded protein and for preparing nucleotide integrases are included for purposes of illustration and are not intended to limit the scope of the invention.

Preparing Nucleotide Integrases By Coexpression of a Group II Intron RNA and a Group II Intron Encoded Protein

Example 1

RNP particles having nucleotide integrase activity and comprising an excised RNA that is encoded by the Ll.ltrB intron of a lactococcal cojugative element pRSO1 of *Lactococcus lactis* and the protein encoded by the ORF of the Ll.ltrB intron were prepared by transforming cells of the BLR(DE3) strain of the bacterium *Escherichia coli,* which has the recA genotype, with the plasmid pETLtrAl9. Plasmid pETLtrAl9, which is schematically depicted in Figure 3, comprises the DNA sequence for the group II intron Ll.ltrB from *Lactococcus lactis,* shown as a thick line, positioned between portions of the flanking exons *ltrB*E1 and *ltr*BE2, shown as open boxes. pETLtrAl9 also comprises the DNA sequence for the T7 RNA polymerase promoter and the T7 transcription terminator. The sequences are oriented in the plasmid in such a manner that the ORF sequence, SEQ. ID. NO. 2, within the Ll.ltrB intron is under the control of the T7 RNA polymerase promoter. The ORF of the Ll.ltrB intron, shown as an arrow box, encodes the protein LtrA. The sequence of the Ll.ltrB

intron and the flanking exon sequences present in pETLtrAl9 are shown in Figure 4 and SEQ. ID. NO. 1. Vertical lines in Figure 4 denote the junctions between the intron and the flanking sequences. The amino acid sequence of the LtrA protein, SEQ. ID. NO. 3 is shown under the ORF sequence, SEQ. ID. NO. 2, in Figure 4. The sequences of EBS1 and EBS2 include nucleotides 457 through 463 (EBS1), nucleotides 401 through 406 (EBS2a) , and nucleotides 367 through 372 (EBS2b). Domain IV is encoded by nucleotide 705 to 2572.

pETLtrAl9 was prepared first by digesting pLE12, which was obtained from Dr. Gary Dunny from the University of Minnesota, with HindIII and isolating the restriction fragments on a 1% agarose gel. A 2.8 kb HindIII fragment which contains the Ll.ltrB intron together with portions of the flanking exons *ltrB*El and *ltrB*E2 was recovered from the agarose gel and the single-stranded overhangs were filled in with the Klenow fragment of DNA polymerase I obtained from Gibco BRL, Gaithersburg, MD. The resulting fragment was ligated into plasmid pET-11a that had been digested with XbaI and treated with Klenow fragment. pET-11a was obtained from Novagen, Madison, WI.

pETLtrAl9 was introduced into the *E. coli* cells using the conventional $CaCl_2$-mediated transformation procedure of Sambrook et al. as described in "Molecular Coning A Laboratory Manual", pages 1-82, 1989 . Single transformed colonies were selected on plates containing Luria-Bertani (LB) medium supplemented with ampicillin to select the plasmid and with tetracycline to select the BLR strain. One colony was inoculated into 2 ml of LB medium supplemented with ampicillin and grown overnight at 37°C with shaking. 1 ml of this culture was inoculated into 100 ml LB medium supplemented with ampicillin and grown at 37°C with shaking at 200 rpm until OD_{595} of the culture reached 0.4. Then isopropyl-beta-D-thiogalactoside was added to the culture to a final concentration of 1 mM and incubation was continued for 3 hours. Then the entire culture was harvested by centrifugation at 2,200 x g, 4°C, for 5 minutes. The bacterial pellet was washed with 150 mM NaCl and finally resuspended in 1/20 volume of the original culture in 50 mM Tris, pH 7.5, 1 mM EDTA, 1 mM DTT, and 10% (v/v) glycerol (Buffer A)and 2 mg/ml lysozyme. Bacteria were frozen at -70°C.

To produce a lysate the bacteria were thawed and frozen at -70°C three times. Then 4 volumes of 500 mM KCl, 50 mM $CaCl_2$, 25 mM Tris, pH 7. 5, and 5 mM DTT (HKCTD) were added to the lysate and the mixture was sonicated until no longer viscous, i.e. for about 5 seconds or longer. The lysate was fractionated into a soluble fraction and insoluble fraction

by centrifugation at 14,000 x g, 4°C, for 15 minutes. Then 5 ml of the resulting supernatant, i.e., the soluble fraction, were loaded onto a sucrose cushion of 1.85 M sucrose in HKCTD and centrifuged for 17 hours at 4°C, 50,0000 rpm in a Ti 50 rotor from Beckman. The pellet which contains the RNP particles was washed with 1 ml water and then dissolved in 25 µl 10 mM Tris, pH 8. 0, 1 mM DTT on ice. Insoluble material was removed by centrifugation at 15, 000 x g, 4°C, for 5 minutes. The result is a preparation of partially-purified RNP particles that comprise the excised Ll.ltrB intron RNA and the LtrA protein

The yield of RNP particles was 25 to 50 $O.D._{260}$ units (~ 16 µg protein) per 100 ml culture, with 1 $O.D._{260}$ units of RNPs containing 0.3 to 3 µg LtrA protein. To minimize nuclease activity, the partially-purified RNPs were further purified by an additional centrifugation through a 1.85 M sucrose cushion, as described above.

Example 2

RNP particles having nucleotide integrase activity and comprising the LtrA protein and the excised Ll.ltrB intron RNA were prepared as described in example 1 except the plasmid pETLtrAl9 was used to transform cells of the BL21(DE3) strain of *E. coli*. The transformed cells were fractionated into a soluble fraction and an insoluble fraction as described in Example 1 to provide a preparation of RNP particles having nucleotide integrase activity

Example 3

RNP particles having nucleotide integrase activity and comprising the LtrA protein and the excised Ll.ltrB intron RNA were prepared by transforming cells of the *E. coli* strains BLR(DE3) with pETLtrAl9 as described in Example 1 except that the transformed *E. coli* were grown in SOB medium and shaken at 300 rpm during the 3 hour incubation. The transformed cells were fractionated into a soluble fraction and an insoluble fraction as described in Example 1 to provide a preparation of RNP particles having nucleotide integrase activity

Example 4

RNP particles having nucleotide integrase and comprising the LtrA protein and the excised Ll.ltrB intron RNA were prepared as described above in sample 1 except that the plasmid pETLtrAl9 was used to transform cells of the *E. coli* strain BL21(DE3). The cells

were also transformed with plasmid pOM62 which is based on the plasmid pACYC184 and has an approximately 150 bp insert of the *argU(dnaY)* gene at the *Eco*RI site. The *argU* gene encodes the tRNA for the rare arginine codons AGA and AGG. The LtrA gene contains 17 of the rare arginine codons. The transformed cells were grown in SOB medium and fractionated into a soluble fraction and an insoluble fraction as described in Example 1 to provide a preparation of RNP particles having nucleotide integrase activity.

Example 5

RNP particles having nucleotide integrase and comprising the excised Ll.ltrB intron RNA and the LtrA protein were prepared by transforming host cells as described above in Example 1 except that the LtrA ORF was tagged at the C-terminus with a His_6 affinity tag and an epitope derived from the Herpes simplex virus glycoprotein D. The tag is used to facilitate isolation of the RNP particles. The plasmid adding the tags was made in two steps by using PCR. In the first step, a fragment containing exon 1 and the LtrA ORF was amplified using primers LtrAexl.Xba having the sequence 5' TCACCTCATCTAGACATTTTCTCC 3', SEQ. ID. NO. 5 which introduces an *Xba* I site in exon 1 of LtrB, and LtrAexpr3 5'CGTTCGTAAAGCTAGCCTTGTGTTTATG 3', SEQ. ID. *NO.* 6, which substitutes a CGA (arginine) codon for the stop codon and introduces an *Nhe* I site at the 3' end of the LtrA ORF. The PCR product was cut with *Xba*I *and Nhe* I, and the restriction fragments gel purified and cloned into pET-27b(+), cut with *Xba* I and *Nhe I* obtained from Novagen, Madison, WI. The resulting plasmid pIntermediate-C fuses the 3' end of the LtrA ORF to an HSV tag and His_6 purification tag, both of which are present on the vector pET-27b(+). In a second step, intron sequences 3' to the ORF and exon 2 are amplified using pLE12 as a template and the 5' primer LtrAConZnl, having the sequence 5'CACAAGTGATCATTTACGAACG 3', SEQ. ID. No. 7 and the 3' primer LtrAex2, which has the sequence 5'TTGGGATCCTCATAAGCTTT GCCGC 3', SEQ. ID. NO. 8. The PCR product is cut with *Bcl*I *and Bam*HI, the resulting fragment filled in, gel purified and cloned into pIntermediate-C, which has been cleaved with *Bpu*1102I and filled in. The resulting plasmid is designated pC-hisLtrAl9.

Cells of the BLR(DE3) strain of *E. coli* were transformed as described in example 1 with pIntermediate-C and cultured at 37°C for 3 hours in SOB medium as described in example 3. The cells were also fractionated into a soluble fraction, which contains RNP

particles having nucleotide integrase activity, and an insoluble fraction as described in example 1. The RNP particles were further purified as described in example 1.

EXAMPLE 6

RNP particles having nucleotide integrase activity and comprising an excised Ll.ltrB intron RNA and the LtrA protein were prepared by transforming host cells as described above in example 1 except that the LtrA ORF was tagged at the N-terminus with a His_6 affinity tag and the epitope tag XPRESS™ which was obtained from Invitrogen, San Diego, CA. The tag is used to facilitate isolation of the RNP particles. The plasmid adding the tags was made in two steps by using PCR. In the first step, a fragment was made in two steps by using PCR mutagenesis. In the first step, the LtrA ORF and 3' exon were amplified and *Bam*Hl sites were appended to both the 5' an 3' end of the LtrA ORF using pLE12 as a substrate and the following pair: 5' primer N-LtrA 5', having the sequence 5'CAAAGGATCCGATGAAACCA ACAATGGCAA 3', SEQ. ID. NO. 9; and the 3' primer LtrAex2, SEQ. ID. *NO.* 8. The PCR product was cut with *Bam*Hl and the resulting restriction fragment was gel purified and cloned into the *Bam*Hl site of plasmid pRSETB obtained from Invitrogen, San Diego, CA. The resulting plasmid pIntermediate-N fuses the N terminus of the LtrA ORF to a His_6 purification tag, and adds an XPRESS™ epitope tag from the vector. In a second step, the 5' exon and Ll.ltrB intron sequences 5' to the ORF were amplified using pLE12 as a substrate and the 5' primer NdeLTR5, having the sequence 5'AGTGGCTTCCATATGCTTGGTCATCACCTCATC 3', SEQ. ID. No. 10 and 3' primer NdeLTR3', which has the sequence 5' GGTAGAACCATATGAAATTCCTCCTCCCTAATCAATTTT 3', SEQ. ID. NO. 11. The PCR product was cut with *Nde* I, the fragment gel purified and cloned into pIntermediate-N, which had also been cut with *Nde* I. Plasmids were screened for the orientation of the insert, and those oriented such that the 5' exon was proximal to the T7 promoter were used to transform the host cells. The resulting plasmid pFinal-N expresses a message under the control of the T7 polymerase promoter which comprises the El and E2 portions of the exons 1 *LtrB*El and *LtrB*E2, and the LtrA ORF fused at the 5'end with an His_6 purification tag and the XPRESS™ epitope tag.

Cells of the BLR(DE3) strain of *E. coli* were transformed as described in example 1 with pIntermediate-N and cultured at 37°C f or 3 hours in SOB medium as described in

example 3. The cells were also fractionated into a soluble fraction, which contains RNP particles having nucleotide integrase activity, and an insoluble fraction as described in example 1. The RNP particles were further purified as described in example 1.

EXAMPLE 7

RNP particles having nucleotide integrase activity and comprising an excised Ll.ltrB intron RNA and the LtrA protein were prepared as described by transforming host cells as described above in example 1 except that the LtrA ORF was tagged at the C-terminus with an intein from *Saccharomyces cerevisiae* VMA1 gene and the chitin binding domain (CBD) from *Bacillus circulans* . The tag was used to facilitate purification of the RNP particles and was added using components of the Impact™ purification system obtained from New England Biolabs, Beverly, MA. A plasmid adding the tags was made in two steps by using PCR. In the first step, the LtrA ORF was amplified by PCR using pETLtrA19 as template and using 5' primer LtrAexpr, 5'-AAACCTCCATATGAAACCAACAATG-3', SEQ. ID. NO. ___and 3' primer ltrimpact: 5'TAACTTCCCGGGCTTGTGTTTATGAATCAC-3', SEQ. ID. NO. ____ which deletes the termination codon and introduces a *Sma*I site. The PCR product was cut with *Nde*I and *Sma*I and cloned into pCYB2, obtained from New England Biolabs, Beverly, MA, and cleaved with the same enzymes. Colonies were screened for inserts and two independent colonies with the desired insert were retained to yield pLI1PInt21 and pLI1PInt22. In a second step, pLI1PInt21 was cleaved with *Pst*I, the overhangs repaired with T4 DNA polymerase in the presence of 0.2 mM dNTPs. The DNA was then phenol extracted, ethanol precipitated and then partially digested with *Pml* I. The approximately 1580 bp *Pml*I- *Pst* I fragment was cloned into pETLtrAl9 digested with *Pml* I. The clones with correct insert were screened and one oriented such that the intein is fused to the C terminus of the LtrA ORF was called pLI1Int. The resulting construct expresses the Ll.ltrB intron and fuses the LtrA ORF with the sequences that encode VMAI intein and CBD.

Cells of the BLR(DE3) strain of *E. coli* were transformed as described in example 1 with pL1Int. The transformants were restreaked on ampicillin selective plates and single colonies were inoculated into 50 mL of LB medium and grown overnight at 37° C. This culture was used to inoculate 0.5 liters of SOB in 4 liter flasks at a 1:100 dilution. The cultures were grown to an OD_{595} 0.7-1.0 and induced with 1mM IPTG at room temperature for 4 hours. The cultures were harvested, washed with 150 mM NaC1 10mM Tris-HCl (pH

7.5), and repelleted and stored in 50 ml of Buffer I (20 mM Tris-HCl (pH8.0), 0.5 M NaCl, 0.1 mM EDTA, 0.1% NP-40). The cells were broken by sonicating for 1 minute 3 times in a Bronson sonicator at setting 7. The lysate was cleared by centrifugation at 12,000 x g for 30 minutes. The cleared lysate was loaded on a chitin affinity column equilibrated with Buffer I. The RNP particles comprising a tagged protein are retained on the column. Then 15 ml of elution buffer (Buffer I+30 mM DTT) was passed through the column, the column flow was stopped, and the column incubated overnight at 4° C to allow self-cleavage of the intein tag and release of the purified RNP particles from the chitin. Flow was restarted and the RNP particles comprising an excised Ll.ltrB intron RNA and the LtrA protein were collected.

EXAMPLE 8

RNP particles having nucleotide integrase activity and comprising the LtrA protein and an excised Ll.ltrB intron RNA having an altered EBS1 sequence were prepared as described above in example 1 except that the cells were transformed and the RNP particles were made using pLI1-EBS1/-6C. The pLI1-EBS1/-6C construct which has a single nucleotide change G to C at position 6 in the EBS1 (G3137C as based on Mills et al, 1996) sequence of the wild-type intron and a complementary change in the 5' exon at position -6 relative to the 5' splice site to permit splicing was constructed via two PCR steps. In the first step pETLtrA19 was subjected to PCR with primers OP2, 5'-GGATCGAGATCTCGATCCCG, SEQ. ID. NO. ____and IP11: 5'CGCACGT TATCGATGTGTTCAC, SEQ. ID. NO. ___to introduce the single nucleotide change in the exon, and with primers IP4, 5'-TTATGGTTGTCGACTTATCTGTTATC, SEQ.ID.NO.___. and OP1, OP1: 5'-CTTCGAATACCGGTTCATAG, SEQ. ID. NO. ____ to introduce the single nucleotide change in EBS1. The single nucleotide change in the IP4 primer introduces a SalI site in the EBS1 sequence, which was subsequently used to identify the desired clones. The second PCR step was performed using the above two PCR products as Primers and pETLtrA19 DNA linearized with *Bgl*II and *Bam*HI as the template. The second PCR product was reamplified with flanking primers OP2 and OP1 using *Pfu* polymerase from Stratagene and digested with *Bgl*II and *Bsr*GI to yield a 554-bp fragment that was cloned between the *Bsr*GI and *Bgl*II sites of pETLtrA19. The desired clones were identified by digestion with *Hin*dIII and *Sal*I, and the region that had been generated by PCR was sequenced completely to insure that no adventitious mutations had been introduced.

EXAMPLE 9

A partially-purified preparation of the LtrA protein, which is encoded by the ORF of the LI.ltrB intron, using plasmid pETLtrA1-1 was prepared. Plasmid pETLtrA1-1 is a derivative of pETLtrAI9 and lacks exon 1 and the intron sequences upstream of the LtrA ORF. Accordingly, the LtrA ORF is directly downstream of the phage T7 promoter following the Shine-Dalgarno sequence in the plasmid. The plasmid map of pETLtrAI-1 is shown in Figure 5.

pETLtrAI-1 was made by using the polymerase chain reaction to amplify the LtrA ORF using the 5' primer LtrAexpr . SEQ.ID. _____, which introduces an *Nde*I site and 3' primer LtrAex2, SEQ. ID. NO. 8. The PCR product was cut with *Nde*I and *Bam*HI, gel purified on a 1%. agarose gel, and cloned into pET-11a. The inserts of pLE12, pETLtrAI9 and pETLtrA1-1, each of which contain the LtrA ORF are depicted in Figure 6.

pETLtrA1-1 was introduced into cells of the *E. coli* strain BLR(DE3) as described in Example 1 and the transformed cells grown for 3 hours in SOB medium at 37°C as described in Example 3. Thereafter, the cells were lysed and the resulting lysate fractionated into a soluble fraction and insoluble fraction by low speed centrifugation as described in Example 1 to provide fractions containing a partially-purified preparation of LtrA protein.

Preparing Nucleotide Integrases using in vitro-synthesized intron RNA

EXAMPLE 10

RNP particles having nucleotide integrase activity and comprising an excised, LI.ltrB intron RNA which lacks the ORF and an LtrA protein was prepared by mixing an in vitro-synthesized intron RNA with an LtrA protein preparation that was made by digesting the RNP particles prepared as described above in example 1 with micrococcal nuclease (MN). Specifically, 1.0 $O.D_{260}$ of the RNP particle preparation were resuspended in 40 µl of 10 mM Tris, HCl, pH 7.5, 10 mM $MgCl_2$, 2.5 mM $CaCl_2$, 5 mM DTT and incubated with 12 or 36 units of MN from Pharmacia for 10 minutes at 22° C , after which the MN was inactivated by addition of EGTA to 7.5 mM.

The group II intron RNA was generated by in vitro transcription of pLI2-ΔORF. pLI2-ΔORF, which has a large deletion in the intron ORF, was derived from pLI2 by inverse PCR with primers ΔORFa: 5'-GGGGGGGCTAGCACGCGTCGCCACGTAATAAATATCTG GACG, SEQ. ID. NO.___and ΔORFb: 5'-GGGGGGGCTAGCACGCGTTGGGAAATG GCAATG ATAGC, SEQ.ID.NO.___, each containing an *Mlu*I site. The PCR product was digested with *Mlu*I and

self-ligated to generate pLI2-ΔORF, thereby replacing amino acids 40 to 572 of the LtrA ORF with threonine and arginine. The plasmid was linearized with *Bam*HI and transcribed with phage T3 RNA polymerase, and the *in vitro*-synthesized RNA (30 to 50μg) was spliced for 60 min at 42°C in 100 μl of 1 M NH_4Cl, 100 mM $MgCl_2$, and 50 mM Tris-HC1 (pH 7.5). Prior to reconstitution, the RNA was heated to 85 to 90°C for 2 minutes, then stored on ice.

0.05 $O.D._{260}$ units of the MN-treated RNP particles was added to 20 μl of reaction medium containing 50 mM Tris-HCl (pH7.5), 10 mM $MgCl_2$, 10 mM KCl, 5 mM DTT, and 1 μg of the spliced RNA to provide RNP particles having nucleotide integrase activity and comprising a modified, excised, Ll.ltrB intron RNA and an LtrA protein.

EXAMPLE 11.

RNP particles having nucleotide integrase and comprising the LtrA protein and an excised Ll.ltrB intron RNA having a kanamycin resistance gene inserted in domain IV in place of the LtrA ORF were prepared as described above in example 10 except that the RNA component was made using pLI2-ΔORF*kan*^{R}. pLI2-ΔORF*kan*^{R}, which replaces amino acids 39-573 of the LtrA ORF with a *kan*^{R} *gene*, was constructed by cloning the 1,252-bp *Sal*I fragment containing the *kan*^{R} gene from pUK4K (Pharmacia, Piscataway, NJ) into the *Mlu*I site of pL12-ORF by blunt-end ligation after filling in both the *Sal*I and *Mlu*I sites with Klenow polymerase (Life Technologies, Gaithersburg, MD)

Comparative Example A

RNP particles lacking nucleotide integrase activity were prepared as described in Example 1 from cells of the BLR(DE3) strain of *E. coli* that had been transformed with plasmid pET11a, which lacks a group II intron. Accordingly, is these RNP particles do not comprise excised, group II RNA or group II intron-encoded proteins and therefore, do not have nucleotide integrase activity.

Comparative Example B.

RNP particles lacking nucleotide integrase activity were prepared as described in Example 1 from cells of the BLR(DE3) strain of *E. coli* that had been transformed with plasmid pETLtrAl9FS, which comprises the sequence of an LtrA ORF having a frame shift 372 base pairs downstream from the initiation codon of the LtrA ORF. frame. Accordingly, the RNP particles contain a truncated LtrA protein, i.e. an LtrA protein lacking the Zn domain and, therefore, do not have nucleotide integrase activity.

Characterization of the RNP particles of Examples 1 and 2.

A portion of the RNP particle preparation of examples 1 and 2 and comparative examples A and B were subjected to SDS polyacrylamide gel electrophoresis. Staining of the resulting gel with Coomassie Blue permitted visualization of the proteins in each of the fractions. A band of approximately 70 kDa, which corresponds to the predicted molecular weight of the LtrA protein was seen in the lanes containing aliquots of the RNP particles of Examples 1 and 2. This band was absent from the lanes containing the RNP particles prepared from comparative examples A and B. On the basis of the staining intensity of the 70 kDa band, the quantity of LtrA protein in 10 OD_{260} units of RNP particles was estimated to be approximately 3 μg. Thus, RNP particles containing the group II intron-encoded protein LtrA can be prepared by expression of the group II intron L1.ltrB in a heterologous host cell.

The reverse transcriptase activities of the RNP particles of examples 1 and 2 and the RNP particles of comparative examples A and B were assayed by incubating each of the RNP particle preparations with a poly(rA) template and oligo (dT_{18}) as a primer. The RNP particles of examples 1 and 2 exhibited reverse transcriptase activity, while the RNP particles of comparative examples A and B exhibited no reverse transcriptase activity. Thus, the methods described in examples 1 and 2 are useful for preparing RNP particles that have reverse transcriptase activity. The reverse transcriptase activity that is present in nucleotide integrases allows incorporation of a cDNA molecule into the cleavage site of the double stranded DNA which is cut by the nucleotide integrase.

Characterizing the Distribution and Yield of the LtrA Protein

A portion of the insoluble fraction and soluble fraction of the lysates from the cells transformed and cultured according to the methods described in examples 1, 2, 3, 4 and 9 were subjected to SDS polyacrylamide gel electrophoresis. Following electrophoresis, the SDS gels were stained with Coomassie blue to compare the yield of the LtrA protein and the distribution of the 70 kDa LtrA protein prepared by the methods of examples 1, 2, 3, 4 and 9. As shown on the gel, more of the LtrA protein was found in the soluble fraction when the transformed BLR (DE3) cells were grown in SOB medium and shaken at 300 rpm than when the transformed BLR cells were grown in LB medium and shaken at 200 rpm. In addition, the total amount of LtrA protein produced by the transformed BLR cells, that is the amount of LtrA in both the soluble and insoluble fractions, increased when, as described in example 4, a

plasmid comprising the Ll.ltrB intron and a plasmid comprising *argU(dnaY)* gene were both introduced into the host cells, the LtrA protein which was expressed in cells transformed with a plasmid which lacks the 5' segment of the Ll.ltrB.intron, as described in example 9, was significantly more insoluble than the LtrA protein which was expressed in cells transformed with a plasmid that contained the 5'segment of the intron as well as the LtrAORF.

Characterization of the Group II Intron-Encoded Protein Prepared According to the Methods of Examples 5-and 6.

A portion of the insoluble fraction and soluble fractions of the lysates from the cells transformed and cultured according to the methods described in examples 5 and 6 and in comparative examples A and B were subjected to electrophoresis on duplicate SDS-polyacrylamide gels. One of the gels was stained with Coomassie blue and the proteins on the duplicate were transferred to nitrocellulose paper by Western blotting. A primary antibody to the HSV antigen and an alkaline phosphatase-labeled anti-mouse IgG secondary antibody were used in an enzyme-linked immunoassay to identify proteins carrying the HSV epitope or the Xpress™ tag. The anti-HSV antibody and the anti-Xpress™ tag antibody bound to a protein having a molecular weight of approximately 70 kDa, which is close to the calculated molecular weight of the LtrA protein. The HSV tagged LtrA protein and the Xpress™ tagged LtrA protein were found in the soluble and insoluble fractions from cells transformed with pIntermediateC and pIntermediateN but not in the soluble fractions and insoluble fractions of cells transformed with pET27b(+) and pRSETB. Thus, the methods of examples 5 and 6 are useful for preparing an RNP particle comprising a tagged group II intron encoded protein. These assays also demonstrated that the amount of the tagged group II intron-encoded protein present in the soluble fraction, from which the RNP particles are derived, increases when the transformed and induced cells are incubated at 22°C as compared to 37°C. In cells grown at 22°C, the yield of the tagged protein was 0.4 to 2 mg per 1 culture, which is 2 to 5% of the total protein, with about 30% being soluble and 40 to 90% of the soluble protein being recovered in RNP particles (0.3 to 3 μg LtrA protein/$O.D._{260}$). In cells grown at 37°C, a high proportion of the protein was insoluble. However, a significant amount of the tagged LtrA protein that was found in the soluble fraction was present in RNP particles.

Characterization of the Purity and Yield of the Protein in the RNP Particles Prepared According to the Method of Example 7

A portion of the RNP particle preparation of example 7 and comparative examples A and B were subjected to SDS polyacrylamide gel electrophoresis, which was subsequently stained with Coomassie Blue. A band of approximately 70 kDa, which corresponds to the predicted molecular weight of the LtrA protein was seen in the lanes containing aliquots of the RNP particles of Example 7 and was absent from the lanes containing the RNP particles prepared from comparative examples A and B. On the basis of the Bradford protein assays of the column eluant, the quantity of LtrA protein in RNP particles in the eluant from the chitin column was estimated to be approximately 0.5 mg/liter of start culture. The LtrA protein in these RNP particles was approximately 95% pure. Accordingly, the method of claim 7 is highly preferred for making large amounts of highly purified RNP particles having nucleotide integrase activity.

Using the RNP Particles to Cleave Double-Stranded DNA and to Insert Nucleotide Sequences into the Cleavage Site.

Nucleotide integrases are useful for cleaving RNA substrate, single-stranded DNA substrates and one or both strands of a double-stranded DNA substrate, catalyzing the attachment of the excised, group II intron RNA molecule to the RNA substrate, the single-stranded DNA substrate, and to the first strand, i.e. the strand that contains the IBS1 and IBS2 sequence, of the double-stranded DNA substrate. Nucleotide integrases also catalyze the formation of a cDNA molecule on the second strand, i.e. the strand that is complementary to the first strand, of a cleaved double-stranded DNA substrate. Thus, the nucleotide integrases are useful analytical tools for determining the location of a defined sequence in a double-stranded DNA substrate. Moreover, the simultaneous insertion of the nucleic acid molecule into the first strand of DNA permits tagging of the cleavage site of the first strand with a radiolabeled molecule. In addition, the automatic attachment of an RNA molecule onto one strand of the DNA substrate permits identification of the cleavage site through hybridization studies that use a probe that is complementary to the attached RNA molecule. An attached RNA molecule that is tagged with a molecule such as biotin also enables the cleaved DNA to be affinity purified. Moreover, the cleavage of RNA molecules, single stranded DNA molecules, and one or both strands of a double stranded DNA molecule and the concomitant

insertion of a nucleotide sequence into the cleavage site permits incorporation of new genetic information or a genetic marker into the cleavage site, as well as disruption of the cleaved gene. Thus, the nucleotide integrases are also useful for rendering the substrate DNA nonfunctional or for changing the characteristics of the RNA and protein encoded by the substrate DNA.

While RNP particles having nucleotide integrase activity can be used to cleave nucleic acid substrates at a wide range of temperatures, good results are obtained at a reaction temperature from about 30°C to about 42°C, preferably from about 30° to about 37°C. A suitable reaction medium contains a monovalent cation such as Na^+ or K^+, and a divalent cation, preferably a magnesium or manganese ion, more preferably a magnesium ion, at a concentration that is less than 100 mM and greater than 1 mM. Preferably the divalent cation is at a concentration of about 5 to about 20 mM. The preferred pH for the medium is from about 6.0-8.5, more preferably about 7.5-8.0.

Because of its reverse transcriptase activity, the LtrA protein, either in the form of an RNP particle which comprises the LtrA protein or as a free protein, i.e., a protein which is not bound to a group II intron RNA, is also useful for transcribing RNA molecules.

Cleavage of Double Stranded DNA Substrates

A. Cleaving a Double-Stranded DNA Substrate with the RNP Particles of Example 1

0.025 $O.D_{260}$ of the RNP particles of Example 1 and comparative examples A and B were incubated for 20 minutes with 150,000 cpm of each of a 5' and 3' end-labeled double-stranded DNA substrate that comprises the wild-type exon 1 and the wild-type exon 2 junction of the *ltrB* gene. The sequence of the 129 base pair substrate, which comprises the 70 base pair exon 1 and exon 2 junction of the *ltrB* gene, plus sequences of the plasmid is depicted in Figure 7A and SEQ. ID. NO. 4. To verify cleavage, the products were isolated on a 6% polyacrylamide gel.

The substrate which is cleaved by the nucleotide integrase, which comprises the excised Ll.ltrB intron RNA and the LtrA protein, is schematically depicted in Figure 8(a). In addition, the IBS1 and IBS2 sequence of the substrate is shown in figure 8(b). As shown in Figure 8, the IBS1 and IBS2 sequences which are complementary to the EBS sequences of the Lltr.B intron RNA are present in exon 1 of the *ltrB* gene. As depicted in Figure 8, the RNP particles prepared according to the method of example 1 cleaved the sense strand of the substrate at position 0, which is the exon 1 and exon 2 junction, and cleaved the antisense

strand at +9. When the RNP particles prepared according to the method of example 1 were treated with either RNase A/T1 to degrade the RNA in the particles, or with proteinase K to degrade the protein component of the particles prior to incubation of the particles with the substrate, no cleavage of the substrate was observed. These results indicate that both the RNA component and the protein component of the nucleotide integrase are needed to cleave both strands of the substrate DNA.

0.025 $O.D._{260}$ units of the RNP particle preparation of example 1 were reacted with 125 fmoles (150,000 cpm) of the 129 base pair internally-labeled DNA substrate for 20 minutes. To verify cleavage, the products were glyoxalated and analyzed in a 1% agarose gel.

A dark band of radiolabel of approximately 1.0 kb RNA and lighter bands of approximately 0.8, 1.1, 1.4, 1.5, 1.6, 1.9, 2.5, 3.2 were observed on the gel. Pretreatment of the reaction products with RNase prior to separation on the agarose gel resulted in the complete disappearance of these bands. These results indicate that the Ll.ltrB intron RNA was attached to the DNA substrate during reaction of the substrate with the RNP particles of example 1. On the basis of the size of Ll.trB intron, it is believed that the band at 2.5 kb represents the integration of the full length group II intron RNA into the cleavage site of the sense strand. The presence of smaller radiolabeled products on the gel is believed to be due to degradation of the integrated intron RNA by RNases which may be present during purification. The finding that the RNA-DNA products withstand denaturation with glyoxal indicates a covalent linkage between the intron RNA and the DNA substrate.

B. Cleaving Double-Stranded DNA Substrates using Nucleotide Integrases Prepared by the Methods of Examples 8, 10, and 11.

0.025 $O.D._{260}$ units of the RNP particle preparation of examples 10 and 11 were reacted with 125 fmoles (150,000 cpm) of the 129 base pair internally-labeled DNA substrate for 20 minutes. To verify cleavage, the products were glyoxalated and analyzed in a 1% agarose gel. To verify that the RNA component of the nucleotide integrase had been partially or fully integrated into the cleavage site, sequences of the exon 1 DNA-intron RNA and exon 2 DNA junctions were analyzed by RT-PCR. The RNP particles prepared as described in examples 10 and 11 were able to efficiently cleave the double-stranded DNA substrate and to either partially or fully integrate the intron RNA subunit of the nucleotide integrase into the cleavage site. Thus, RNP particles that comprise LtrA protein and an Ll.ltrB intron RNA which lacks an ORF sequence have complete nucleotide integrase activity. Similarly RNP

particles that comprise an LtrA protein and an LltrB intron RNA in which the ORF has been replaced with a sequence encoding a different gene product also have complete nucleotide integrase activity

0.025 $O.D._{260}$ units of the RNP particle preparations of example 8 were reacted with 125 fmoles (150,000 cpm) of the 129 base pair internally-labeled double-stranded DNA substrate which comprises the sequence depicted in Figure 7A for 20 minutes. In addition, 0.025 $O.D._{260}$ units of the RNP particle preparations of example 8 were reacted with 125 fmoles (150,000 cpm) of a 129 base pair internally-labeled double-stranded DNA substrate which comprises a modified exon 1 and wild-type exon 2 of the Ll.ltrB gene for 20 minutes. The sequence of the first strand of the 129 base pair substrate, in which the nucleotide at position -6 relative to the putative cleavage site in the wild-type exon 1 is changed from a C to a G is underlined in Figure 7B. The putative cleavage sites in the first strand of the substrates shown in Figure 7A and 7B are depicted by a vertical line. To verify cleavage, the products were glyoxalated and analyzed in a 1% agarose gel. Endonuclease assays were also conducted to confirm that cleavage occurred between nucleotides -1 an +1 in the first strand of the substrate and at position +9 in the second strand of the substrate, and also to confirm that a nucleic acid molecule had been inserted into the cleavage site. The RNP particles prepared as described in example 8 were able to efficiently cleave the double-stranded DNA substrate shown in Figure 7b and to either partially or fully integrate the intron RNA subunit of the RNP particles into the cleavage site. The EBS1 sequence of the modified Ll.ltrB intron in the RNP particles prepared as described in example 8 is complementary to the IBS1 sequence of the substrate shown in Figure 7b. The RNP particles prepared as described in example 8, however, were not able to efficiently cleave the substrate depicted in Figure 7a. The EBS1 sequence of the modified Ll.ltrB intron in the RNP particles prepared as described in example 8 is not complementary to the IBS1 sequence of the substrate shown in Figure 7a. These results indicate that changing the EBS1 sequence of a group II intron RNA alters the target site specificity of the nucleotide integrase that comprises the modified group II intron RNA.

CLAIMS

What is Claimed is:

1. A method of preparing RNP particles having nucleotide integrase activity comprising the steps of:

(a) providing an isolated, excised, group II intron RNA;

(b) providing a group II intron-encoded protein; and

(c) incubating the excised, group II intron RNA with the group II intron-encoded protein to provide an RNP particle comprising the excised, group II intron RNA bound to the group II intron-encoded protein.

2. The method of claim 1 wherein the group II intron-encoded protein of step (b) is obtained by a process comprising the following steps:

a) expressing a DNA molecule which comprises an open reading frame sequence that encodes said group II intron-encoded protein in a host cell to provide an RNP particle comprising said group II intron-encoded protein bound to an RNA molecule;

b) lysing said host cell to obtain said RNP particle; and

c) removing said RNA molecule from said group II intron-encoded protein.

3. The method of claim 2 wherein the DNA molecule lacks an intron sequence upstream of said open reading frame sequence.

4. The method of claim 3 wherein said open reading frame sequence is operably linked to a promoter.

5. The method of claim 2 wherein said RNP particle is obtained from a soluble fraction of the lysed host cell.

6. The method of claim 2 wherein the DNA molecule further comprises a nucleotide sequence encoding a tag for facilitating isolation of the RNP particle.

7. The method of claim 6 wherein the nucleotide sequence which encodes the tag is at the 5' end or the 3' end of the open reading frame sequence.

8. The method of claim 2 wherein the RNA is removed from the group II intron-encoded protein by contacting the RNP particle with a nuclease.

9. The method of claim 1 further comprising the step of introducing the DNA molecule into a heterologous host cell prior to step (a).

10. The method of claim 1 wherein the group II intron-encoded protein is provided by a process comprising the following steps

a) expressing a DNA molecule which encodes a wild-type or a modified group II intron RNA into a host cell to provide an RNP particle comprising said group II intron-encoded protein bound to an RNA molecule;

b) lysing said host cell to provide said RNP particle; and

c) removing said RNA molecule from said group II intron-encoded protein.

11. The method of claim 10 wherein the DNA molecule encodes a splicing-defective group II intron RNA.

12. The method of claim 10 wherein the RNP particle is obtained from a soluble fraction of the lysed cell.

13. The method of claim 10 further comprising the step of introducing the DNA molecule into a heterologous host cell prior to step (a).

14. The method of claim 1 wherein the isolated, excised, group II intron RNA is a wild-type group II intron RNA.

15. The method of claim 1 wherein the isolated, excised, group II intron RNA is a modified group II intron RNA.

16. The method of claim 15 wherein the modified group II intron RNA comprises a modification in the loop region of domain IV.

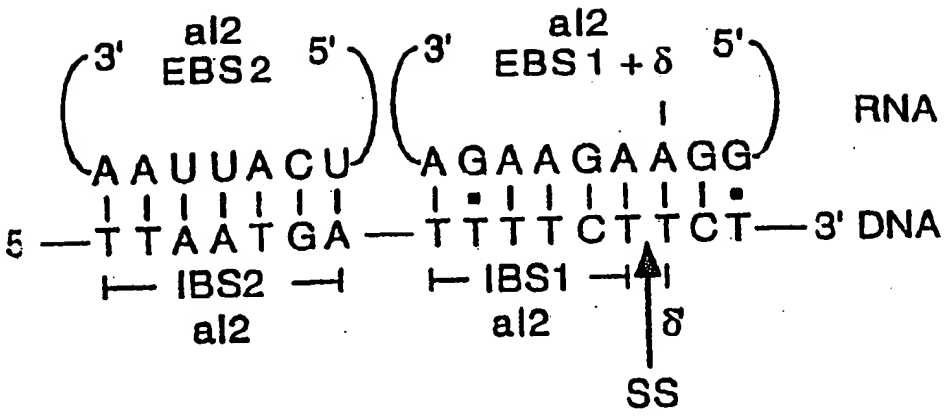
17. The method of claim 15 wherein the modified group II intron RNA has a modified EBS1 sequence.

18. The method of claim 15 wherein the modified group II intron RNA has a modified EBS2 sequence.

19. The method of claim 1 wherein said isolated group II intron RNA comprises a first hybridizing sequence capable of hybridizing with a first intron RNA binding sequence on one strand of a DNA substrate and a second hybridizing sequence capable of hybridizing with a second intron RNA binding sequence on said one strand of the DNA substrate.

20. The method of claim 16 wherein said isolated group II intron RNA further comprises a delta nucleotide that is complementary to a delta prime nucleotide on said one strand of the substrate, said delta prime nucleotide being located at position +1 relative to a cleavage site on said one strand of said DNA substrate.

Fig. 1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3' EBS2 5'
al2
3' EBS1 + δ 5'
RNA
A A U U A C U
A G A A G A A G G
5 — T T A A T G A — T T T T C T T C T — 3' DNA
IBS2
IBS1
al2
al2
δ
SS

Fig. 2

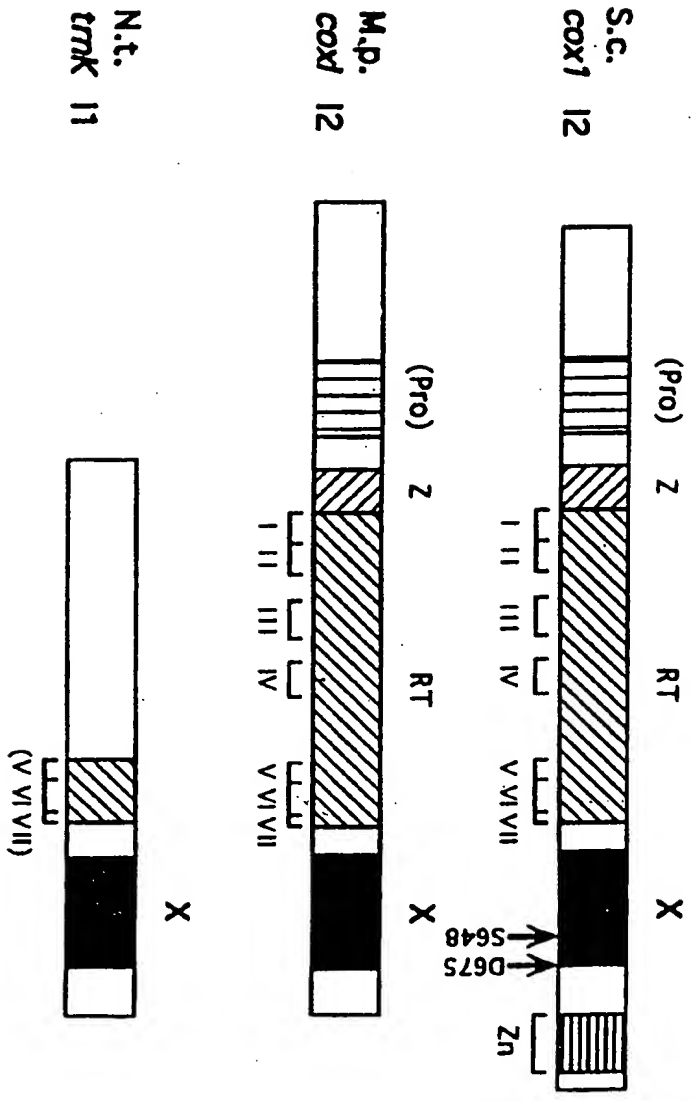
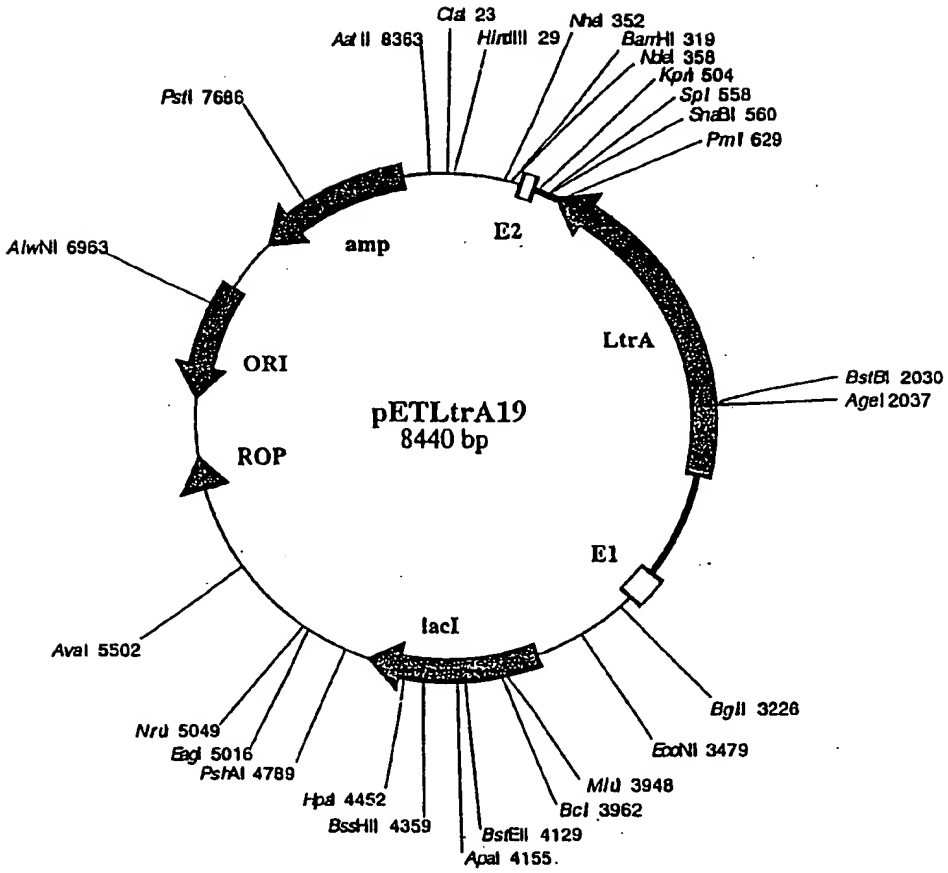

N.t. tmK 11
M.p. cox1 I2
S.c. cox1 I2
(Pro)
Z
I
II
III
IV
V VI VII
RT
X
(V VI VII)
D67S
S648
Zn

Fig. 3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AatII 8363
HindIII 29
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NheI 358
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PstI 7686
E2
amp
AlwNI 6963
LtrA
ORI
pETLtrA19
8440 bp
BstBI 2030
AgeI 2037
ROP
E1
lacI
AvaI 5502
BglII 3226
NruI 5049
EagI 5016
PshAI 4789
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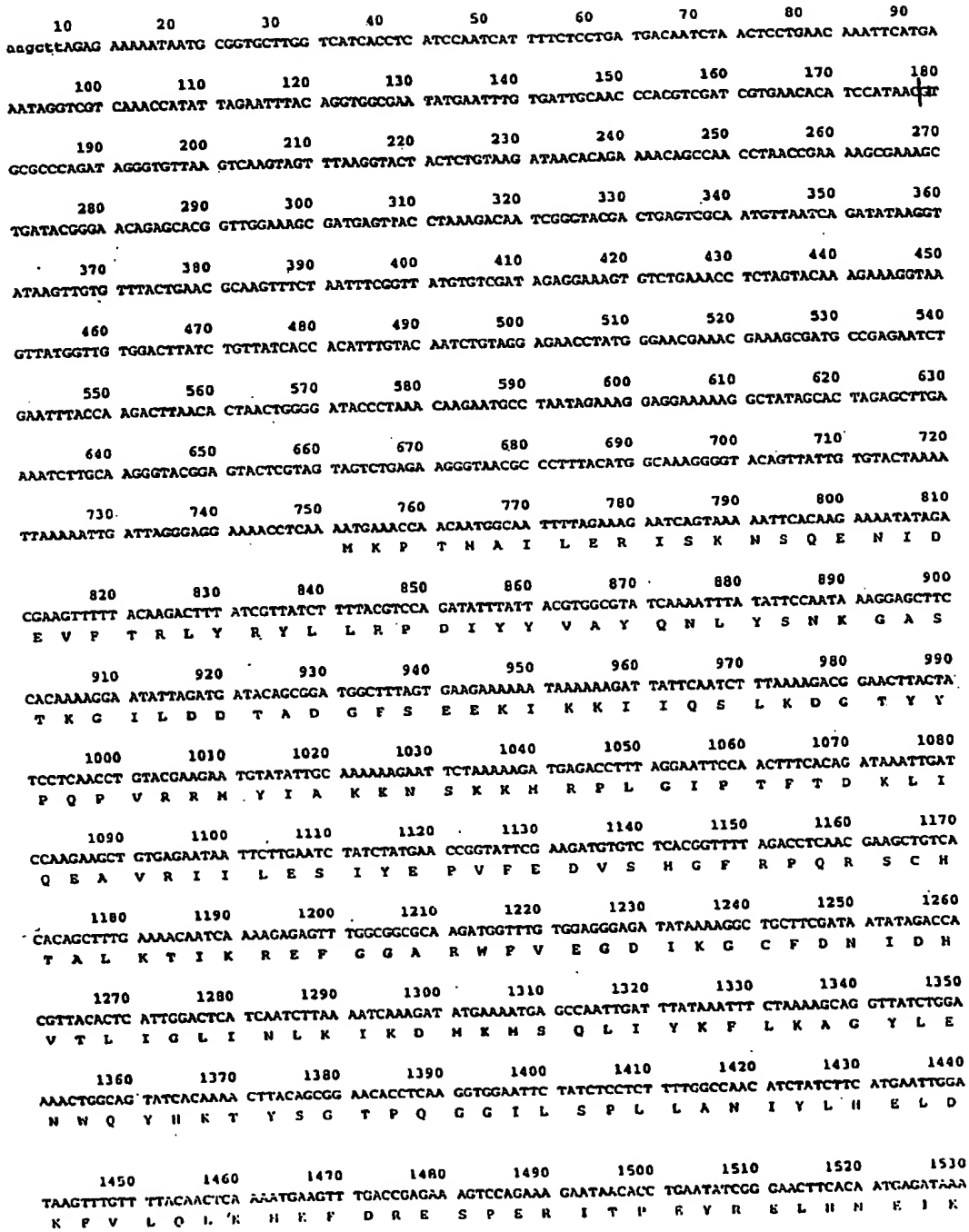
Fig. 4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AATAGGTCGT CAAACCATAT TAGAATTTAC AGGTGGCGAA TATGAATTTG TGATTGCAAC CCACGTCGAT CGTGAACACA TCCATAAGTT
190 200 210 220 230 240 250 260 270
GCGCCCAGAT AGGGTGTTAA GTCAAGTAGT TTAAGGTACT ACTCTGTAAG ATAACACAGA AAACAGCCAA CCTAACCGAA AAGCGAAAGC
280 290 300 310 320 330 340 350 360
TGATACGGGA ACAGAGCACG GTTGGAAAGC GATGAGTTAC CTAAAGACAA TCGGGTACGA CTGAGTCGCA ATGTTAATCA GATATAAGGT
370 380 390 400 410 420 430 440 450
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460 470 480 490 500 510 520 530 540
GTTATGGTTG TGGACTTATC TGTTATCACC ACATTTGTAC AATCTGTAGG AGAACCTATG GGAACGAAAC GAAAGCGATG CCGAGAATCT
550 560 570 580 590 600 610 620 630
GAATTTACCA AGACTTAACA CTAACTGGGG ATACCCTAAA CAAGAATGCC TAATAGAAAG GAGGAAAAAG GCTATAGCAC TAGAGCTTGA
640 650 660 670 680 690 700 710 720
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730 740 750 760 770 780 790 800 810
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M K P T N A I L E R I S K N S Q E N I D
820 830 840 850 860 870 880 890 900
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E V P T R L Y R Y L L R P D I Y Y V A Y Q N L Y S N K G A S
910 920 930 940 950 960 970 980 990
CACAAAAGGA ATATTAGATG ATACAGCGGA TGGCTTTAGT GAAGAAAAAA TAAAAAAGAT TATTCAATCT TTAAAAGACG GAACTTACTA
T K G I L D D T A D G F S E E K I K K I I Q S L K D G T Y Y
1000 1010 1020 1030 1040 1050 1060 1070 1080
TCCTCAACCT GTACGAAGAA TGTATATTGC AAAAAAGAAT TCTAAAAAGA TGAGACCTTT AGGAATTCCA ACTTTCACAG ATAAATTGAT
P Q P V R R M Y I A K E N S K K H R P L G I P T F T D K L I
1090 1100 1110 1120 1130 1140 1150 1160 1170
CCAAGAAGCT GTGAGAATAA TTCTTGAATC TATCTATGAA CCGGTATTCG AAGATGTGTC TCACGGTTTT AGACCTCAAC GAAGCTGTCA
Q E A V R I I L E S I Y E P V F E D V S H G F R P Q R S C H
1180 1190 1200 1210 1220 1230 1240 1250 1260
CACAGCTTTG AAAACAATCA AAAGAGAGTT TGGCGGCGCA AGATGGTTTG TGGAGGGAGA TATAAAAGGC TGCTTCGATA ATATAGACCA
T A L K T I K R E F G G A R W F V E G D I K G C F D N I D H
1270 1280 1290 1300 1310 1320 1330 1340 1350
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V T L I G L I N L K I K D M K M S Q L I Y K F L K A G Y L E
1360 1370 1380 1390 1400 1410 1420 1430 1440
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N W Q Y H K T Y S G T P Q G G I L S P L L A N I Y L H E L D
1450 1460 1470 1480 1490 1500 1510 1520 1530
TAAGTTTGTT TTACAACTCA AAATGAAGTT TGACCGAGAA AGTCCAGAAA GAATAACACC TGAATATCGG GAACTTCACA ATGAGATAAA
K F V L Q L K H E F D R E S P E R I T P E Y R E L H N E I K

Fig. 4 (con't.)

```
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 R  I  S  H  R  L  K  K  L  E  G  E  E  K  A  K  V  L  L  E  Y  Q  E  K  R  K  R  L  P  T

      1630       1640       1650       1660       1670       1680       1690       1700       1710
ACTCCCCTGT ACCTCACAGA CAAATAAAGT ATTGAAATAC GTCCGGTATG CGGACGACTT CATTATCTCT GTTAAAGGAA GCAAAGAGGA
 L  P  C  T  S  Q  T  N  K  V  L  K  Y  V  R  Y  A  D  D  F  I  I  S  V  K  G  S  K  E  D

      1720       1730       1740       1750       1760       1770       1780       1790       1800
CTGTCAATGG ATAAAAGAAC AATTAAAACT TTTTATTCAT AACAAGCTAA AAATGGAATT GAGTGAAGAA AAAACACTCA TCACACATAG
 C  Q  W  I  K  E  Q  L  K  L  P  I  H  N  K  L  K  M  E  L  S  E  E  K  T  L  I  T  H  S

      1810       1820       1830       1840       1850       1860       1870       1880       1890
CAGTCAACCC GCTCGTTTTC TGGGATATGA TATACGAGTA AGGAGAAGTG GAACGATAAA ACGATCTGGT AAAGTCAAAA AGAGAACACT
 S  Q  P  A  R  F  L  G  Y  D  I  R  V  R  R  S  G  T  I  K  R  S  G  K  V  K  K  R  T  L

      1900       1910       1920       1930       1940       1950       1960       1970       1980
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 N  G  S  V  E  L  L  I  P  L  Q  D  K  I  R  Q  F  I  F  D  K  K  I  A  I  Q  K  K  D  S

      1990       2000       2010       2020       2030       2040       2050       2060       2070
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 S  W  F  P  V  H  R  K  Y  L  I  R  S  T  D  L  E  I  I  T  I  Y  N  S  E  L  R  G  I  C

      2080       2090       2100       2110       2120       2130       2140       2150       2160
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 N  Y  Y  G  L  A  S  N  F  N  Q  L  N  Y  F  A  Y  L  M  E  Y  S  C  L  K  T  I  A  S  K

      2170       2180       2190       2200       2210       2220       2230       2240       2250
ACATAAGGGA ACACTTTCAA AAACCATTTC CATGTTTAAA GATGGAAGTG GTTCGTGGGG CATCCCGTAT GAGATAAAGC AAGGTAAGCA
 H  K  G  T  L  S  K  T  I  S  M  F  K  D  G  S  G  S  W  G  I  P  Y  E  I  K  Q  G  K  Q

      2260       2270       2280       2290       2300       2310       2320       2330       2340
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 R  R  Y  F  A  N  F  S  E  C  K  S  P  Y  Q  P  T  D  E  I  S  Q  A  P  V  L  Y  G  Y  A

      2350       2360       2370       2380       2390       2400       2410       2420       2430
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 R  N  T  L  E  N  R  L  K  A  K  C  C  E  L  C  G  T  S  D  E  N  T  S  Y  E  I  H  H  V

      2440       2450       2460       2470       2480       2490       2500       2510       2520
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 N  K  V  K  N  L  K  G  K  E  K  W  E  H  A  M  I  A  K  Q  R  K  T  L  V  V  C  F  H  C

      2530       2540       2550       2560       2570       2580       2590       2600       2610
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 H  R  H  V  I  H  K  H  K  *

      2620       2630       2640       2650       2660       2670       2680       2690       2700
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      2710       2720       2730       2740       2750       2760       2770
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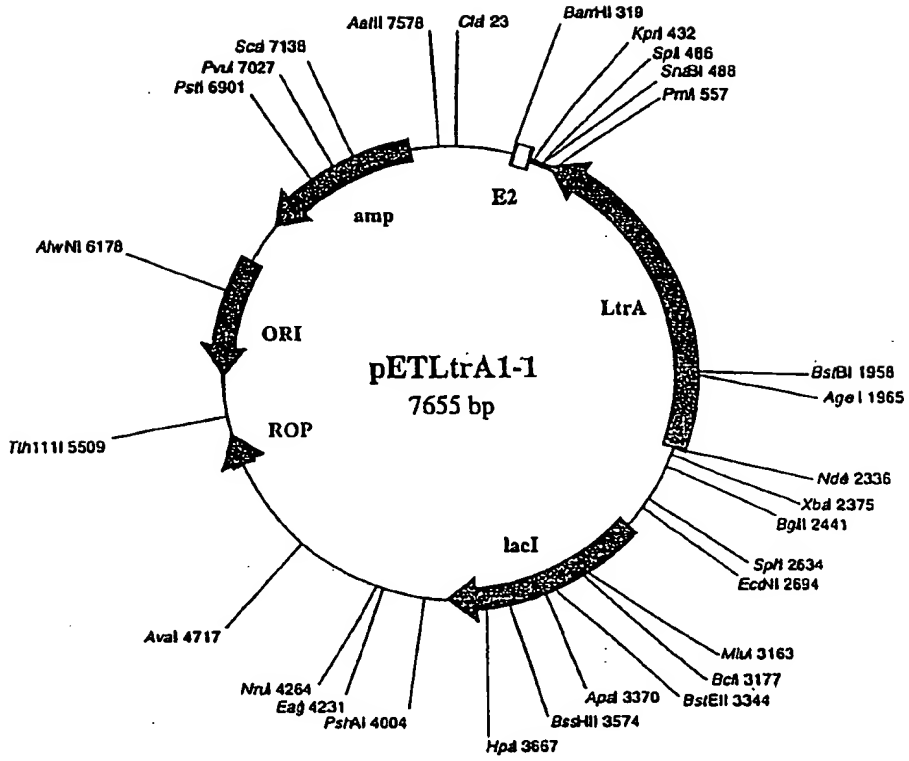
Fig. 5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ORI
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7655 bp
BstBI 1958
AgeI 1965
ROP
Tth111I 5509
NdeI 2336
XbaI 2375
BglII 2441
lacI
SphI 2534
EcoNI 2694
AvaI 4717
MluI 3163
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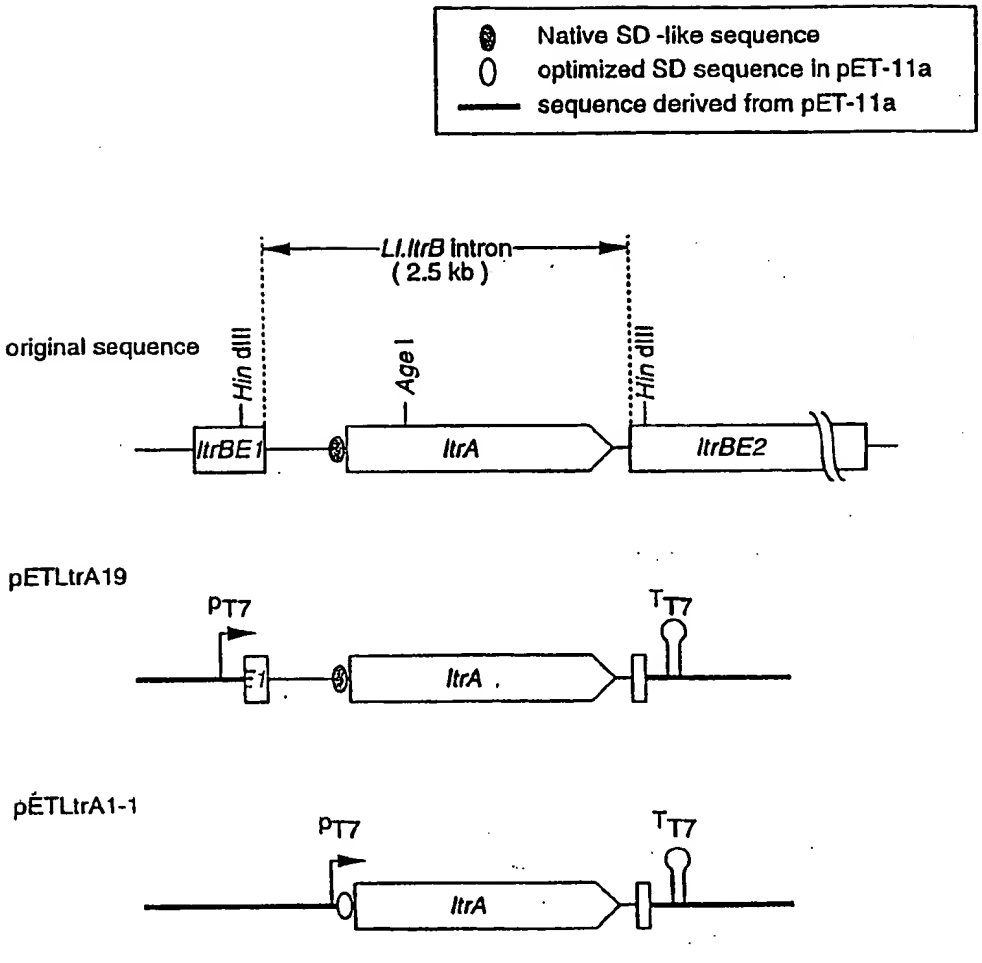
Fig. 6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optimized SD sequence in pET-11a
sequence derived from pET-11a
LI.ltrB Intron
(2.5 kb)
original sequence
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Hin dIII
ltrBE1
ltrA
ltrBE2
pETLtrA19
pT7
TT7
ltrA
pETLtrA1-1
pT7
TT7
ltrA

Fig. 7

A. Wild-type 129 bp dsDNA substrate

cgctctagaactagtggatccTTGCAACCCACGTCGATCGTGAACACATCCATAAC|CATATCATTTTTAA
gcgagatcttgatcacctaggAACGTTGGGTGCAGCTAGCACTTGTGTAGGTATTG|GTATAGTAAAAATT

TTCTACGAATCTTTATACTGGgaattcgatatcaagcttatcgataccgtcgacctcga 129
AAGATGCTTAGAAATATGACCcttaagctatagttcgaatagctatggcagctggagct

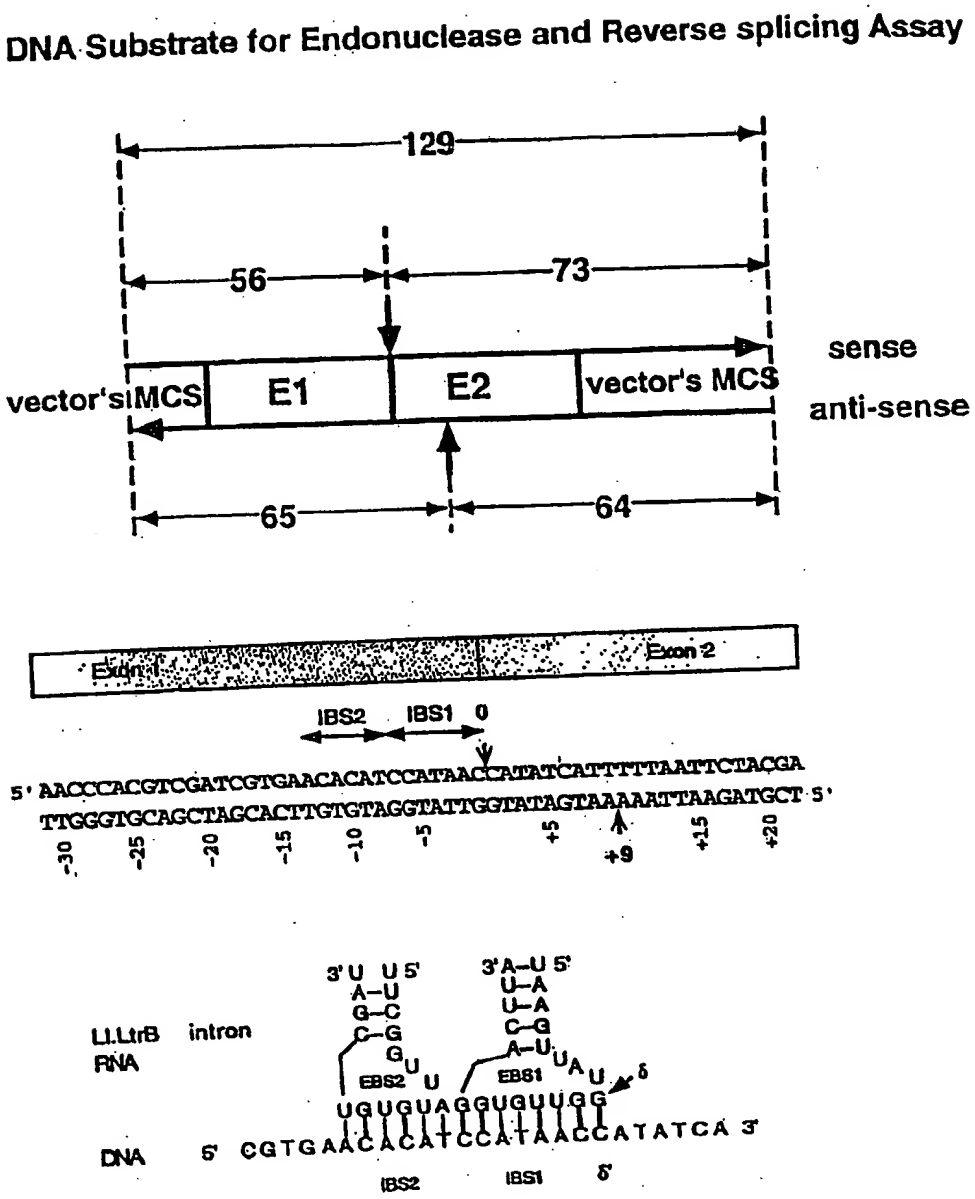
B. C-6G 129 bp dsDNA substrate

cgctctagaactagtggatccTTGCAACCCACGTCGATCGTGAACACATCGATAAC|CATATCATTTTTAA
gcgagatcttgatcacctaggAACGTTGGGTGCAGCTAGCACTTGTGTAGCTATTG|GTATAGTAAAAATT

TTCTACGAATCTTTATACTGGgaattcgatatcaagcttatcgataccgtcgacctcga 129
AAGATGCTTAGAAATATGACCcttaagctatagttcgaatagctatggcagctggagct

UPPER CASE: Lactococcus Ll.ltrB exon sequence
lower case: Vector DNA sequence
vertical line: position 0, exon 1 and exon 2 junction
underlined: mutation in the C-6G dsDNA substrate

Fig. 8


DNA Substrate for Endonuclease and Reverse splicing Assay
129
56
73
sense
vector's
MCS
E1
E2
vector's MCS
anti-sense
65
64
Exon 1
Exon 2
IBS2
IBS1
0
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TTGGGTGCAGCTAGCACTTGTGTAGGTATTGGTATAGTAAAAATTAAGATGCT 5'
-30
-25
-20
-15
-10
-5
+5
+9
+15
+20
LI.LtrB intron RNA
EBS2
EBS1
δ
UGUGUAGGUGUUGG
DNA
5' CGTGAACACATCCATAACCATATCA 3'
IBS2
IBS1
δ'

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/10687

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12P 19/34; A01N 43/04; C12Q 1/68; C07H 21/02, 21/04.
US CL :536/24.5; 514/44; 435/6, 91.1, 91.31, 91.51, 91.53.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 536/24.5; 514/44; 435/6, 91.1, 91.31, 91.51, 91.53.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5,698,421 A (LAMBOWITZ et al.) 16 December 1997, see the entire patent.	1-20

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 07 AUGUST 1998	Date of mailing of the international search report 15 SEP 1998
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer Dwitha Lawrence for TEKCHAND SAIDHA Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/10687

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

STN search included the files - Medline, Caplus, Wpids, Biosis, Scisearch & Biotechds.
APS was also conducted. Search terms - group II intron rna, intron encoded protein, RNP, endonuclease?, integrase, dna encoding integras? or endonuclease?, etc.